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RICIN TOXICITY IN BALB/C 3T3 CELLS: CORRELATION OF TOTAL PROTEINS WITH DOSE LEVEL BY MASS SPECTROMETRY AND PROTEOMICS

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14. ABSTRACT
This report is required for the U.S. Army Edgewood Chemical Biological Center In-House Laboratory Independent Research project "Cell Toxicity by NMR and Mass Spectrometry." NMR and/or mass spectrometry (MS) in combination with chemometrics analysis can yield physiological details in the form of metabolome or proteome biomarker information, but model-building with IC_{50}/ED_{50} values obtained from traditional cytotoxicity assays has been required to correlate biomarker concentration with toxicity [S.V. Vulimiri, et al., *Chem. Res. Toxicol.* **2009**, *22*, 492; E.M. Lenz, et al., *J. Pharm. Biomed. Anal.* **2004**, *35*, 599]. We have analyzed cellular protein extracts from BALB/c 3T3 murine fibroblasts dosed with three ricin concentrations. Using a new method whereby no adjustment is made for cell concentration as toxin dose increases, we show that the average number of cellular extract proteins identified by MS and the calculated percent inhibition are each related logarithmically, but in an inverse manner, to ricin concentration. In addition, the average number of proteins identified by MS is related linearly to the calculated percent inhibition. These results indicate that, if at least eight ricin concentrations are included as is done for typical dye-based toxicity assays, toxicity could be determined and effective dose values obtained directly from LC-MS/MS data.

15. SUBJECT TERMS
3T3 murine fibroblasts; Cell toxicity; Liquid chromatography; Mass spectrometry; LC-MS; Ricin; *Ricinus communis*

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PREFACE

The work described in this report was authorized under Project No. 61110191A00, In-House Laboratory Independent Research. This work was started in January 2009 and completed in September 2009.

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CONTENTS

FIGURES

TABLES

RICIN TOXICITY IN BALB/C 3T3 CELLS: CORRELATION OF TOTAL PROTEINS WITH DOSE LEVEL BY MASS SPECTROMETRY AND PROTEOMICS

1. INTRODUCTION

Traditional cell toxicity assays lead to an IC_{50} or EC_{50} value based on the determination of cell death. However, additional information is desirable for understanding a toxin's total effects. Nuclear magnetic resonance (NMR) and/or mass spectrometry (MS) in combination with chemometrics analysis can yield physiological details in the form of metabolome or proteome biomarker information. However, model-building with IC_{50}/ED_{50} values obtained from traditional cytotoxicity assays has been required to correlate the biomarker concentration with toxicity [1, 2]. We hypothesize that NMR or MS can be used for the direct determination of EC_{50} or IC_{50} in addition to biomarker identification. Here, we report on a study to explore this hypothesis for MS using liquid-chromatography and tandem mass spectrometry (LC-MS/MS) with ricin dosed BALB/c 3T3 murine fibroblasts as a model system. An MS-based chemometrics procedure is also expected to have advantages including: 1) no dyes or labeled compounds and 2) high sample throughput.

2. METHODS

Note: Ricin is extremely toxic. Its use is controlled under the Biological Select Agents and Toxins Program in the United States by the Centers for Disease Control and Prevention (Atlanta, GA). Handling of ricin should follow strict safety procedures determined in collaboration with the safety office of the research laboratory's organization.

2.1 Ricin Preparation.

Ricin communis agglutinin II (ricin, Vector Laboratories, Burlingame, CA) was dialyzed into 10 mM sodium phosphate buffer (pH 7.0, PB) over a period of ~ 24 h with gentle stirring using three volumes of ~ 600 mL PB. Dialysis was carried out on ice using regenerated cellulose or cellulose ester Dispodialyzers® (Spectrum Laboratories, Rancho Dominguez, CA) with 5000 or 8000 molecular weight cutoff. Dialyzed ricin was stored at 0 – 4 °C. The dialyzed ricin concentration was determined at 25 °C by ultraviolet (UV) absorbance using either a JASCO Model J-810 Spectropolarimeter (JASCO Analytical Instruments, Easton, MD) equipped with a PTC-423S Peltier thermoelectric temperature control system or a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, Inc., Thermo Fisher Scientific, Waltham, MA). For the JASCO method, 15 absorbance measurements on one aliquot of the solvent blank solution (no protein) were recorded at intervals of 1 s and averaged. Fifteen measurements were then recorded on one aliquot of the ricin sample and averaged. The blank average was subtracted from the sample average. The concentration was calculated using Beer's Law with $E^{0.1\%}_{280nm} = 1.4$ [3]. For the Nanodrop® method, blank-subtracted measurements were recorded on an aliquot of the ricin sample. The concentration was calculated using Beer's Law with $E^{0.1\%}_{280nm} = 1.4$ [3]. The Nanodrop® measurement procedure and concentration calculation procedure were carried out three times, each using fresh aliquots of blank and ricin sample, and the concentration results averaged. The results of the two procedures were within error of each other. The dialyzed ricin was sterile filtered prior to use with the cell cultures and the concentration verified by the Nanodrop® method.

2.2 Ricin Exposure of BALB/c 3T3 Murine Fibroblasts.

The fibroblasts (CCL-163 American Type Culture Collection [ATCC], Manassas, VA) were passaged a minimum of three times after thawing prior to testing. Cell culture flasks (75 cm^2) were seeded at 8 x 10^3 cells/cm^2 and maintained in culture at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 h prior to treatment. Exposures were performed 24 ± 2 h after seeding the 75-cm^2 flasks. Three concentrations of ricin were prepared by diluting the dialyzed ricin in cell culture medium. The concentrations corresponded to IC_{20}, IC_{50}, and IC_{80} concentrations previously determined by Neutral Red Uptake (NRU) assay [4, 5]. Six replicate sets of flasks (2 flasks per set) were prepared per ricin concentration. In addition, flasks were prepared containing untreated cells (Vehicle Controls, VC), and containing media with and without ricin. After ricin exposure, the flasks were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for another 48 h.

Cell harvesting was carried out 48 h ± 0.5 hr post-exposure. Medium from each set of flasks (two flasks per concentration of ricin and vehicle controls) was removed. The attached cells remaining in the flasks were rinsed twice with Hank's Balanced Salt Solution (HBSS) and then incubated at room temperature for 3 min with trypsin-versine (Product # 17-161E, Lonza Walkersville, Inc., Walkersville, MD). The trypsin-versine was neutralized with the addition of 37 °C Routine Culture Medium (RCM) containing serum. The cells were removed from the flasks and centrifuged for 5 min at 1200 rpm. The supernatant was then discarded, and the pellet was resuspended in 4 mL of 37 °C PBS. A 700-µL sample was removed for analyses not reported here. The remaining cells were centrifuged for 5 min at 1200 rpm. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of 37 °C PBS and then frozen at -80 °C.

2.3 Liquid-Chromatography/Mass Spectrometry Sample Preparation.

The cell samples were thawed and lysed by ultrasonication (25 s on, 5 s off, 4 min total) using a Branson Digital Sonifier® (Danbury, CT). The lysate was centrifuged at 14,000 rpm for 20 min at 10 °C using a Beckman GS-15R centrifuge and F2402H Rotor. The supernatant was transferred to a Microcon® YM-3 filter unit (Millipore, Billerica, MA) and centrifuged at 14,000 rpm and 10 °C (500 µL for 30 min x 2, total volume of 1.00 mL). The filtrate was stored at -20 °C for other analyses. The cellular proteins in the retentate were denatured overnight at 37 °C with 300 µL of 7.2 M urea and 3 µg/mL dithiothreitol in 100 mM ammonium bicarbonate (ABC). The urea was removed by centrifugation (14,000 rpm, 30 min, RT) and the retentate was washed with 200 µL ABC followed by centrifugation using an Eppendorf centrifuge (5415C with rotor F-45-18-11 or 5415D with rotor F-45-24-11, Eppendorf North America, Westbury, NY) at 14,000 rpm for 30 min (RT). The filter unit was then transferred to a new receptor tube, and the proteins in the retentate were digested at 37 °C for 7 hr with 5 µL sequencing grade trypsin (Product # 511A, Promega, Madison, WI) in 10 µL acetonitrile and 235 µL ABC. The tryptic peptides were isolated by centrifuging at 14,000 rpm, 15 min, RT (Eppendorf 5415C or D). The filtrate containing the tryptic peptides was stored at 4 °C until analysis.

2.4 Liquid-Chromatography/Mass Spectrometry Experiments.

For each sample, 10 µL of filtrate containing tryptic peptides (described above) was diluted with 90 µL of aqueous phase (95% H_2O, 5% acetonitrile). Ten microliters of this dilution was injected into a Thermo Electron Corporation Finnigan Surveyor HPLC (Thermo

Scientific, Waltham, MA), and the peptides were separated using a 0.1 x 150 mm C_{18} Hypersil GOLD KAPPA column (5 μ particle size, 175 Å pore size, Fisher Scientific International, Pittsburgh, PA) with a linear gradient of 20 to 80% organic phase (100% acetonitrile, 0.1% formic acid). The column was connected to a Finnigan LTQ tandem ion trap MS fitted with a nanospray ESI source operated at 1.82 kV with a collision energy of 25 V. A data-dependent mode and scan range of *m/z* 300-2000 were used. The full mass spectra were collected, followed by MS on the resulting five most intense ions.

2.5 Proteomics Analysis.

A 3T3 protein database was constructed in a FASTA format using the annotated proteome sequences from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov, accessed July 27, 2010). For this task, an in-house PERL (http://www.activatestate.com/Products/ActivePerl, accessed July 27, 2010) program was used to automatically download the annotated 3T3 proteome sequences from the NCBI. The database was constructed by translating putative protein-encoding genes and contains amino acid sequences of potential tryptic peptides obtained by the *in silico* digestion of all 3T3 proteins, assuming up to two missed cleavages. The acquired mass spectra were searched against this database with the SEQUEST algorithm (Thermo Scientific). The SEQUEST thresholds for searching the product ion mass spectra were Xcorr, deltaCn, Sp, RSp, and deltampep. These parameters provide a uniform matching score of all candidate peptides. The generated output files of these candidate peptides were validated using the PeptideProphet™ algorithm (http://peptideprophet.sourceforge.net, accessed July 27, 2010). Peptide sequences with probability scores of 95% and higher were retained.

3. RESULTS AND DISCUSSION

The LC-MS/MS experiments were carried out on 24 cellular protein extract samples as described above and summarized in Table 1. Typical LC-MS/MS data for a single sample is shown in Figure 1.

Table 1. Summary of Tryptic Peptide Samples Analyzed by LC-MS/MS

Sample Type	Ricin Concentration (μg/mL)	# of Replicate Samples
Vehicle Control Cells	---0---	6
Cells dosed with IC_{20} concentration of ricin	9.54×10^{-04}	6
Cells dosed with IC_{50} concentration of ricin	2.15×10^{-03}	6
Cells dosed with IC_{80} concentration of ricin	5.97×10^{-03}	6
Total	----	24

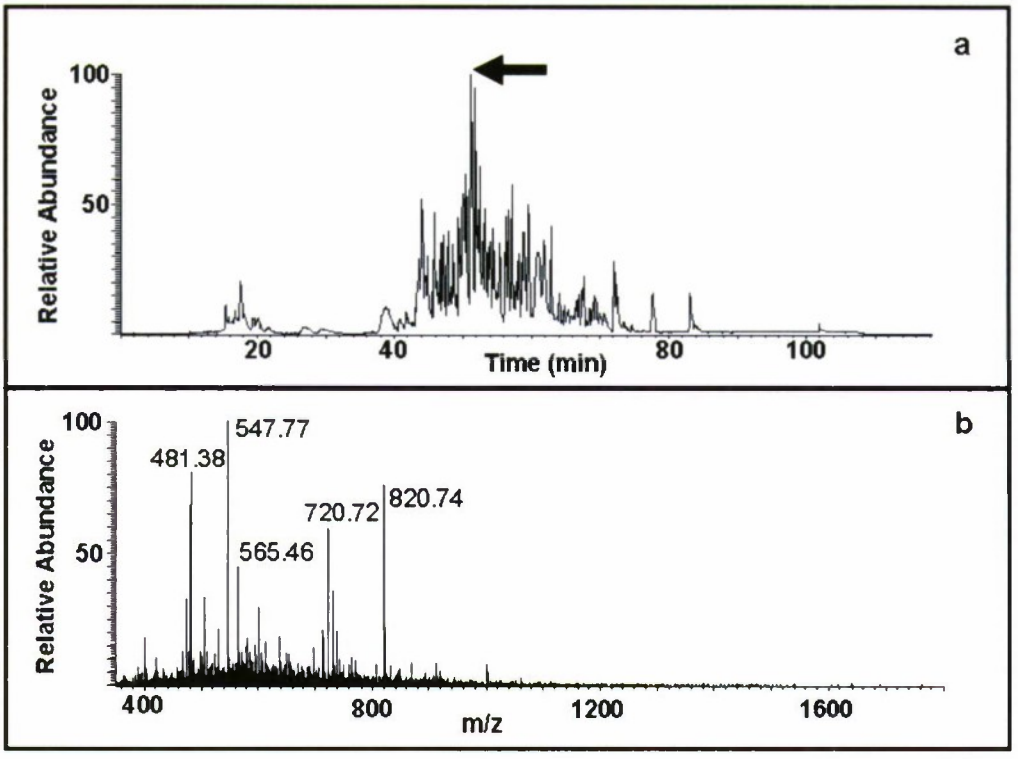

Figure 1. Liquid Chromatography-Mass Spectrometry Data from Extracted Cellular Proteins. a) Total ion chromatogram (The arrow marks the peak having a 51-min retention time.), and b) Full MS spectrum for LC peak with 51-min retention time.

For this experiment, the attached cells recovered from each set of cell growth flasks (representing a single ricin dose) were suspended in 1 mL of PBS (see Section 2.2). As the toxin dose and thus the percent inhibition are increased, the result will be fewer total cells recovered and correspondingly fewer total cells per milliliter suspended in PBS. It is expected that the total amount of recoverable protein per milliliter would be dependent on the total number of cells. The signal-to-noise of the LC-MS/MS data from the tryptic peptides would therefore decrease corresponding with toxin dose, resulting in fewer proteins identified by proteomics analysis and forming the basis for toxicity analysis. Table 2 provides the average number of proteins identified by MS for each ricin concentration. The relative ratios for the proteins could be determined per sample type and protein expression relative to ricin dose could be examined using regression analysis. However, that type of analysis is more appropriately carried out after first adjusting either the cell concentration so that the total cells per sample from which proteins are extracted is constant or the volume of cellular protein extract injected for LC-MS/MS analysis to take into account the decrease in total cells in the original 1 mL of PBS. An analysis along this line is in progress, but is outside the scope of this report. For this report, we focus on the relationship of the total number of proteins identified relative to toxin dose.

Table 2. Average Number of Proteins Identified from Recovered Cellular Proteins

Cell Sample Type	Average # of Proteins Identified	Normalized Average # of Proteins Identified
Vehicle Control	47.3	100.0
Dosed with IC_{20} concentration of ricin	39.2	82.8
Dosed with IC_{50} concentration of ricin*	30.6	56.7
Dosed with IC_{80} concentration of ricin	13.8	29.2

*Average of 5 (rather than 6) replicate samples. One sample was not included for the IC_{50} set due to a Microcon filter unit membrane malfunction that occurred during extraction.

As noted under Section 2.2, the ricin concentrations used for dosing corresponded to IC_{20}, IC_{50}, and IC_{80} concentrations that were calculated from previously carried out NRU assay experiments [3]. Because the percent inhibition was not actually measured here, we will refer to percent inhibition values for these ricin doses as "calculated percent inhibition" values. A plot of the normalized average number of proteins identified versus ricin dose mirrors that of calculated percent inhibition versus ricin dose (Figure 2). Each parameter (normalized average number of proteins identified and calculated percent inhibition) is related logarithmically to ricin concentration. As would be expected based on the results in Figure 2, a plot of the average number of proteins identified versus the calculated percent inhibition (Figure 3) fits a linear curve ($R^2 = 0.998$) having a negative slope.

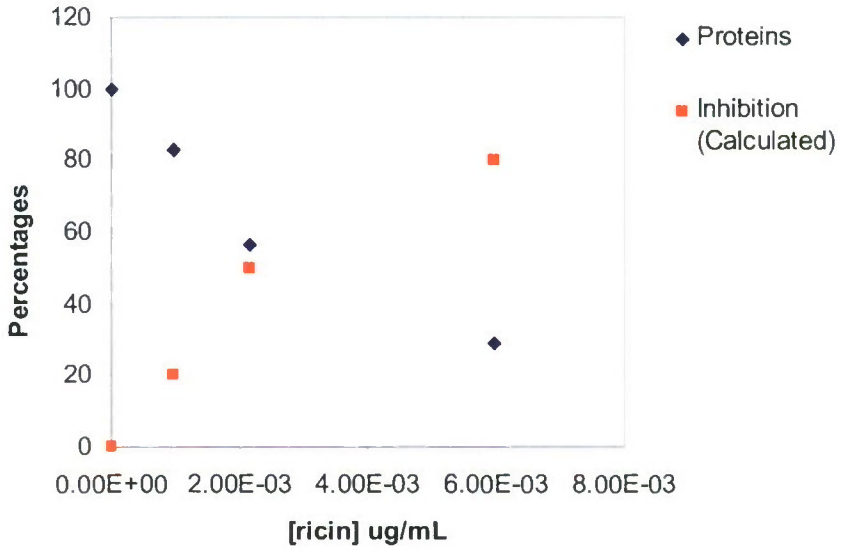

Figure 2. Calculated Percent Inhibition (orange squares) and Normalized Average Number of Proteins Identified (blue diamonds) Versus Ricin Dose

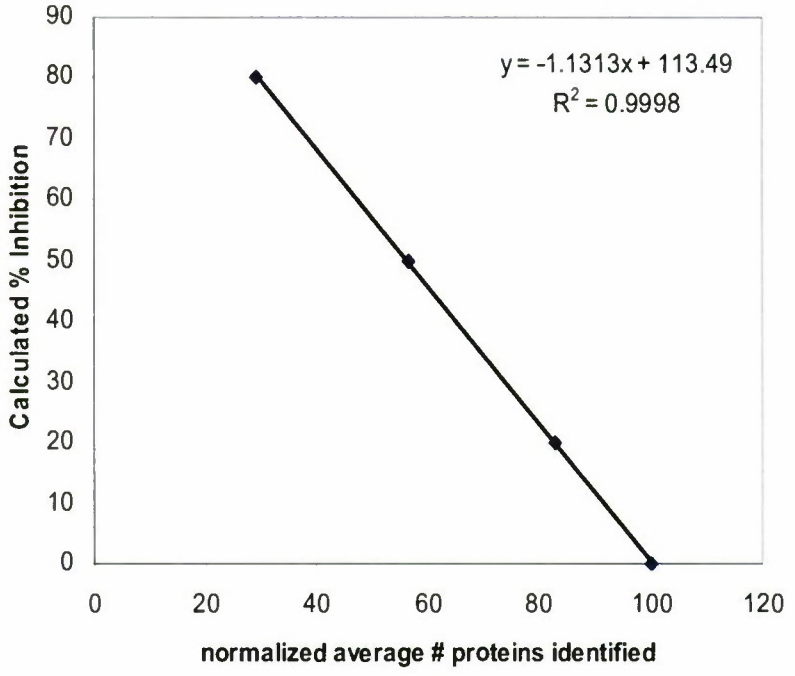

Figure 3. Calculated Percent Inhibition Versus Normalized Average Number of Proteins Identified by LC-MS/MS of Cellular Protein Extracts from Ricin Dosed Cells

4. CONCLUSIONS

Proteomics studies of dosed cells typically involve adjusting the cell concentration so that it is constant across all samples immediately prior to protein extraction, or adjusting the volume of protein extract injected (for liquid-chromatography and tandem mass spectrometry [LC-MS/MS] analysis) according to the cell concentration. These procedures are carried out to keep the overall MS signal-to-noise level constant from one sample to the next. The proteins are then identified and individual proteins quantitated in search of specific biomarkers related to dosing. The analysis requires model-building with IC_{50}/EC_{50} values obtained independently from cytotoxicity assays such as a Neutral Red Uptake (NRU) assay. Here we have analyzed cellular protein extracts from BALB/c 3T3 murine fibroblasts dosed with ricin. The cell concentration per dose was constant at the initial time of dosing. However, at the time for sample collection (48 hr post dosing) only attached cells were harvested and no adjustment was made for cell concentration. This procedure takes advantage of the decrease in MS signal-to-noise for cellular protein extracts that is expected as inhibition of cell growth increases with toxin dose. The assumption is that as the intensity for individual proteins drops below the limit of detection, fewer proteins are identified from samples that contained fewer cells at the time of harvest. Using this procedure, we have shown that the average number of cellular extract proteins identified and the calculated percent inhibition are each related logarithmically, but in an inverse manner, to ricin concentration. We have also shown that the average number

of proteins identified is related linearly to the calculated percent inhibition. These results indicate that, if at least eight ricin concentrations are included as is done for the NRU assay [4, 5], toxicity could be determined and effective dose values obtained directly from LC-MS/MS data. We are planning to carry out such an experiment. If the results are in agreement with the three-concentration study carried out here, they will be validated by measuring toxicity of the ricin preparation independently using the NRU assay. If the validation is successful, it will prove that LC-MS/MS can be used for the direct determination of EC_{50} or IC_{50} in addition to biomarker identification, as originally hypothesized.

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